Signal Transducer and Activator of Transcription (STAT)-3 Activates Nuclear Factor (NF)-κB in Chronic Lymphocytic Leukemia Cells

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Abstract

Nuclear factor (NF)-κB plays a major role in the pathogenesis of B-cell neoplasms. A broad array of mostly extracellular stimuli has been reported to activate NF-κB, to various degrees, in chronic lymphocytic leukemia (CLL) cells. Because CLL cells harbor high levels of unphosphorylated (U) signal transducer and activator of transcription (STAT)-3 protein and U-STAT3 was reported to activate NF-κB, we sought to determine whether U-STAT3 activates NF-κB in CLL. Using the electrophoretic mobility shift assay (EMSA) we studied peripheral blood low-density cells from 15 patients with CLL and found that CLL cell nuclear extracts from all the samples bound to an NF-κB DNA probe, suggesting that NF-κB is constitutively activated in CLL. Immunoprecipitation studies showed that STAT3 bound NF-κB p65, and confocal microscopy studies detected U-STAT3/NF-κB complexes in the nuclei of CLL cells, thereby confirming these findings. Furthermore, infection of CLL cells with retroviral STAT3-shRNA attenuated the binding of NF-κB to DNA, as assessed by EMSA, and downregulated mRNA levels of NF-κB-regulated genes, as assessed by quantitative polymerase chain reaction. Taken together, our data suggest that U-STAT3 binds to the NF-κB p50/p65 dimers and that the U-STAT3/NF-κB complexes bind to DNA and activate NF-κB-regulated genes in CLL cells.

Introduction

Chronic lymphocytic leukemia (CLL) is the most common adult leukemia in the Western Hemisphere. CLL is characterized by a dynamic imbalance between the proliferation and apoptosis of neoplastic B-lymphocytes co-expressing CD5 and CD19 antigens, leading to the accumulation of these cells in the peripheral blood, bone marrow, and lymphatic tissues (1). Several mechanisms have been reported to provide CLL cells with a survival advantage. One such mechanism involves the activation of nuclear factor (NF)-κB (2-8). NF-κB plays

an important role in the survival and proliferation of normal and neoplastic B cells. In CLL, NF-κB has been found to be activated, to a variable degree, regardless of disease stage or treatment status (2-8), suggesting that NF-κB might be a target for therapy in CLL (9-11).

The NF-κB family of transcription factors consists of p50, p52, p65 (RelA), c-Rel, and RelB, which share an N-terminal Rel homology domain responsible for DNA binding and homo- and heterodimerization. NF-κB dimers bind to κB binding sites within the promoters/ enhancers of NF-κB target genes and regulate transcription. Typically, NF-κB dimers are associated with one of three IκB proteins, IκBα, IκBβ, or IκBε, or the precursor proteins p100 and p105, which maintain NF-κB dimers in the cytoplasm in an inactive state. The activation of NF-κB can be mediated by either the canonical pathway or the alternative pathway. The canonical pathway is mainly activated by extracellular factors such as ligands, whose interaction with their corresponding cellular receptors results in activation of the β subunit of the IκB kinase (IKK) complex (IKKβ) that induces the phosphorylation and degradation of the NF-κB inhibitor IκBα. NF-κB activation in response to antigen receptor ligation is mediated by the IKK enzymatic subunits, IKKα, IKKβ, and IKKγ (NEMO). Following IκBα degradation, NF-κB heterodimers (composed of p50, p65, and/or c-Rel) translocate to the nucleus and bind to DNA. Activation of the alternative NF-κB pathway results from the transformation of NF-κB2/p100 to p52, which is triggered by the phosphorylation of NF-κB2/p100 by the α-subunit of the IKK complex (IKKα). This allows for nuclear translocation of p52, along with RelB, and induction of NF-κB-regulated genes (12).

NF-κB is activated in CLL by several different mechanisms: interaction with stroma cells (13), activation of the tumor necrosis factor receptor (TNFR) family members (14-17), activation of the cell surface receptor CD40 by its ligand CD154 (4, 18, 19), activation of the B-cell receptor (20), activation of Notch signaling (21), induction of nitric oxide synthase (22), deregulation of the caspase-recruitment domain (CARD) membrane-associated guanylate kinase protein 1(23), deregulation of the T cell leukemia/lymphoma-1 (TCL1) oncogene (24), induction of glycogen synthase kinase-3β (GSK-3β) (25), and modulation of epigenetic regulators (26).

In 2005, Yang et al. described another, previously unknown, mechanism of NF-κB activation (27). They found that unphosphorylated (U) signal transducer and activator of transcription (STAT)-3 binds to the NF-κB dimers p65/p50 in competition with IκB. The U-STAT3/NF-κB complex translocates to the nucleus, binds to DNA, and activates NF-κB-regulated genes. We recently found that STAT3, constitutively phosphorylated on serine 727 residues in CLL cells, induces the production of STAT3 protein and that CLL cells harbor high levels of U-STAT3 (28). Therefore, we sought to determine whether, as in other cellular systems (29), U-STAT3 activates NF-κB in CLL cells.

Materials and Methods

Cell fractionation

After obtaining Institutional Review Board (IRB)-approved informed consent, we collected peripheral blood (PB) cells from healthy donors and from 22 patients with CLL who were

treated at The University of Texas MD Anderson Cancer Center Leukemia Clinic during the years 2006-2010. The clinical characteristics of the patients with CLL whose PB samples were used in the current study are presented in Table 1. To isolate low-density cells, PB cells were fractionated using Ficoll Hypaque 1077 (Sigma-Aldrich, St. Louis, MO). More than 90% of the CLL PB cells were CD19+/CD5+ lymphocytes. To isolate healthy volunteers' CD19+ cells, low-density PB cells were fractionated using micro-immunomagnetic beads (Miltenyi Biotec, Auburn, CA) in accordance with the manufacturer's instructions. Flow cytometry analysis confirmed that 95% of the fractionated cells were CD19+.

Electrophoretic mobility shift assay (EMSA)

Non-denatured nuclear extracts were prepared using the NE-PER extraction kit (Thermo Scientific, Rockford, IL). Two micrograms of nuclear protein were incubated with a biotin-labeled NF-κB p65 binding-site DNA probe (5′-AGTTGAGGGGACTTTCCCAGGC-3′; synthesized by Sigma Genosys, The Woodlands, TX) in binding buffer for 30 minutes on ice. Following incubation, the samples were separated on a 5% polyacrylamide gel in Tris-borate EDTA, transferred onto a nylon membrane, and fixed on the membrane by ultraviolet cross-linking. The biotin-labeled probe was detected with strepavidin-horseradish peroxidase (Gel–Shift Kit; Panomics, Fremont, CA). As a negative control, we used a probe lacking nuclear extracts. The competition control consisted of up to 7-fold excess unlabeled cold probe combined with the biotin-labeled probes.

Immunoprecipitation

Cell pellets were lysed in ice-cold radioimmunoprecipitation (Ripa) assay buffer containing 1mM sodium orthovanadate, 10 mM sodium fluoride, 100 μM ethylenediaminetetraacetic acid (EDTA), 5 mM β-glycerol phosphate, 1 μg/mL aprotinin, 0.4 mM benzamidine, 1 μg/mL antipain, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 μg/mL leupeptin, and 1 μg/mL trypsin inhibitor soybean. The lysed cell pellets were incubated on ice for 5 minutes and vortexed for 1 minute. Then, cell debris was eliminated by centrifugation at 14,000 rpm for 15 minutes, supernatants were harvested, and the protein concentration was determined using the Micro BCA protein assay reagent kit (Thermo Scientific, Pierce, Rockford, IL). Whole-cell lysates were incubated with 4 μg of polyclonal rabbit anti–human STAT3 antibodies (Upstate Cell Signaling Solutions/Millipore, Billerica MA) for 16 hours at 4°C. Protein A agarose beads (Upstate Cell Signaling Solutions) were added for 2 hours at 4°C. As a negative control, whole-cell lysates were incubated with either rabbit serum and protein A agarose beads or protein A agarose beads only. After 3 washes with Ripa assay buffer, the beads were resuspended in sodium dodecyl sulfate (SDS) sample buffer, boiled for 5 minutes, and removed by centrifugation. The supernatant proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (as described below in "Western blot analysis") and subsequently probed with mouse anti-human NF-κB P65 antibodies (Cell Signaling Technology, Beverly, MA).

Western blot analysis

Western immunoblotting was performed as previously described (28). Briefly, cell pellets were lysed in ice-cold Ripa assay buffer, incubated on ice for 5 minutes, and vortexed for 1

minute. Then, cell debris was eliminated by centrifugation at 14,000 rpm for 15 minutes, supernatants were harvested, and the protein concentration was determined using the Micro BCA protein assay reagent kit (Thermo Scientific). Supernatant proteins were denatured by boiling for 5 minutes in SDS, separated by SDS-PAGE using either 5% or 10% density gels, and transferred to a nitrocellulose membrane. After transfer, equal loading was verified by Ponceau staining.

The membranes were blocked with 5% skim milk in Tris-buffered saline and incubated with the following antibodies: monoclonal mouse anti–human STAT3 (BD Biosciences, San Jose, CA); monoclonal mouse anti–human phosphotyrosine STAT3 and polyclonal rabbit anti–human phosphoserine STAT3 (Cell Signaling Technology); and monoclonal mouse anti-human NF-κB p50 (Pierce Biotechnology, Rockford IL) and p65 (Sigma-Aldrich). After binding with horseradish peroxidase-conjugated secondary antibodies, blots were visualized with an enhanced chemiluminescence detection system (GE Healthcare, Piscataway, NJ), and densitometry analysis was performed using an Epson Expression 1680 scanner (Epson America, Long Beach, CA). Densitometry values were normalized by dividing the numerical value of each sample signal by the numerical value of the signal from the corresponding loading control. In some experiments, the membranes were stripped by incubation with stripping buffer (62.5 mM Tris-HCl, pH 6.7, 2% SDS, 100 mM β-mercaptoethanol) for 30 minutes at 50°C, washed, and reprobed with an antibody.

Confocal microscopy

CLL low-density cells were cytospun on poly-L-lysine–coated slides and fixed in 3.7% formaldehyde for 15 minutes at room temperature on a shaker. The slides were then washed 3 times with PBS, incubated with 1% Triton X-100 for 5 minutes at room temperature, and washed 3 more times with PBS before blocking with mouse serum for 1 hour. After blocking, the slides were washed in PBS and incubated overnight with PE-labeled mouse anti–human -STAT3 antibody (BD Biosciences) and AlexaFluor 488-labeled mouse anti-human NF-κB (BioLegend, San Diego, CA). After incubation, the slides were washed three times in PBS and then mounted with Vectashield hard set (Vector Laboratories, Burlingame, CA). The mounted slides were viewed using an Olympus FluoView 500 laser scanning confocal microscope (Olympus America, Houston, TX), and the images were analyzed using FluoView software (Olympus).

Generation of green fluorescence protein (GFP)-lentivirus STAT3 shRNA and infection of cells

293T cells (American type culture collection) were co-transfected with green fluorescence protein (GFP)-lentivirus STAT3 small hairpin RNA (shRNA) or GFP-lentivirus empty vector and the packaging vectors pCMVδR8.2 and pMDG (generously provided by Dr. G. Inghirami, Torino, Italy) using the superfect transfection reagent (Qiagen, Inc., Valencia, CA). 293T cell culture medium was changed after 16 hours and collected after 48 hours. The culture medium was filtered through a 45-μm syringe filter to remove floating cells; the lentivirus was then concentrated by filtration through an Amicon ultra centrifugal filter device (Milipore), and the concentrated supernatant was used to infect CLL cells.

CLL cells (5×10^6/mL) were incubated in 6-well plates (Becton Dickinson, Franklin Lakes, NJ) in 2 mL DMEM supplemented with 10% FCS and transfected with 100 μL of the viral supernatant. Polybrene (10 ng/mL) was added to the viral supernatant at a ratio of 1:1000 (v/v). Transfection efficiency was measured after 48-72 hours and was found to range between 30% and 60% (calculated on the basis of the ratio of propidium iodide (PI)-negative/GFP-positive cells). These experiments were conducted by using the FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). Data analysis was performed using CellQuest Pro software (Becton Dickinson). A shift from the control curve was calculated as percent shift beyond control.

Chromatin Immunoprecipitation (ChIP)

The enzymatic ChIP Kit (Cell Signaling Technology) was used. Fractionated CLL cells were cross-linked with 1% formaldehyde for 10 minutes at room temperature, a glycine solution was added to arrest the reaction, and the cells were washed with ice-cold PBS. The cells were pooled, pelleted, and incubated on ice for 10 minutes in cell lysis buffer supplemented with 100 mM phenylmethylsulfonyl fluoride, dithiothreitol (DTT) and a protease cocktail inhibitor mix. Nuclei were pelleted and resuspended in buffer supplemented with DTT, digested by micrococcal nuclease, and homogenized on ice. Following sonication (35 pulses, 20 sec/pulse at 25–30% power) and centrifugation, 12 mg of sheared chromatin was incubated with anti-STAT3 or Rabbit serum (negative control) overnight at 4°C. Then, magnetic-coupled protein G beads were added and the chromatin was incubated for 2 hours in rotation. Antibody-bound protein/DNA complexes were washed, eluted, treated with proteinase digest proteins, and subjected to PCR analyses. An aliquot of chromatin that was not incubated with an antibody was used as the input control sample. The primers to amplify the human STAT3 promoter were F: 3′-CCG AAC GAG CTG GCC TTT CAT-5′ and R: 5′-GGA TTG GCT GAA GGG GCT GTA-3′; to amplify the VEGF C promoter: F: 3′-CCA GAA AGG ATG TGT AGC ATC-5′ and R:5′-TGG TCC TCT GTA ACC TGC TCA-3′; to amplify the CXCR5 promoter: F: 3′-CCT GCC TCA CAA CTC ATC ACT-5′ and R:'-GTT GAG ACA ATT ATT GCC GGG-3′; and to amplify the CCL5 promoter: F: 3′- CTCACACTGTAAATTGAGGCA-5′and R: 5′-AGG TCG CTT AGC AAG TAA ATG-3′. The human RPL30 gene primers were provided by Cell Signaling Technologies. PCR products were resolved on 1.8% agarose gels containing ethidium bromide.

RNA purification and quantitative real-time PCR

RNA was isolated using an RNeasy purification procedure (Qiagen, Inc.). RNA quality and concentration were analyzed with a NanoDrop spectrophotometer (ND-1000; NanoDrop Technologies, Wilmington, Delaware). Ten micrograms of total RNA was used for one-step RT-PCR (Applied Biosystems, Foster City, CA) with the sequence detection system ABI Prism 7700 (Applied Biosystems) using the TaqMan gene expression assay for STAT3, VEGF C, CCL5, and CXCR5 (Hs00374200_m1, Hs01099203_m1, Hs00174575_m1, Hs00173527_m1, respectively), according to the manufacturer's instructions. Samples were run in triplicate, and relative quantification was performed by comparing the values obtained at the fractional cycle number at which the amount of amplified target reaches a fixed (C_T) threshold.

Results

NF-κB is constitutively activated in CLL cells

Several investigators have reported that NF-κB is activated, to various degrees, in CLL cells (2-8). To confirm these observations, we obtained PB CLL cells and, by using EMSA, assessed NF-κB-DNA binding. We studied randomly chosen PB samples from 15 patients with CLL who had favorable or unfavorable prognostic factors and different stages of disease (Table 1). As shown in Fig. 1, we found that nuclear extracts from the PB cells of all 15 patients formed complexes with the NF-κB DNA probe, and the addition of excess (5-fold) unlabeled (cold) probe inhibited the binding. These data suggest that NF-κB was constitutively activated in the CLL cells of all studied patients.

STAT3 binds NF-κB in CLL cells, and the U-STAT3/NF-κB complex translocates to the nucleus

The p65/p50 heterodimer is the prototype NF-κB transcription factor that is crucial for the expression of genes encoding several cytokines and pro-inflammatory mediators. To determine whether STAT3 binds NF-κB in CLL cells, as previously reported in another cellular system (29), we immunoprecipitated STAT3 protein with rabbit anti–human STAT3 antibodies, and detected NF-κB p65 and p50, and STAT3 proteins in the immunoprecipitate by western blotting using mouse anti-human p65, p50, and STAT3 antibodies, respectively. As shown in Fig. 2A, the NF-κB p65 and p50 proteins were detected in CLL cell STAT3 immunopreciptate obtained from 3 different PB CLL samples. To further delineate this observation, p65 protein was immunoprecipitated with mouse anti–human p65 antibodies, and STAT3, serine pSTAT3, p65, and p50 proteins were detected by western blot analysis. As shown in Fig. 2B, p65, p50, STAT3, but not serine pSTAT3 proteins were detected in CLL cell p50 immunoprecipitate obtained from 4 different PB CLL samples, suggesting that U-STAT3 binds NF-κB p50/p65 dimers in CLL cells.

To confirm this observation, we conducted confocal microscopy studies. In normal PB CD19+ cells, faint signals of STAT3 were detected, as we previously reported (28). Scattered signals of the NF-κB p65 protein were detected in the cytoplasm and the nucleus in these control cells (Fig. 3A), likely as a result of the constant shuttling of IκBα/NF-κB complexes between the nucleus and the cytoplasm (30). In contrast, in CLL cells (characterized by an overwhelmingly big nucleus and a thin cytoplasm (28)), dense signals of STAT3 and of the p65 form of NF-κB, as well as STAT3/p65 complexes, were detected mainly in the nucleus and, to a lesser extent, in the cytoplasm (Fig. 3B).

U-STAT3/NF-κB complexes bind to DNA and activate NF-κB-regulated genes

To determine whether U-STAT3/NF-κB complexes bind to DNA we isolated nuclear extracts from CLL cells and, using EMSA, assessed binding of CLL cell nuclear proteins to the κB binding site. As shown in Fig. 4A, we found that both anti-p65 and anti-STAT3 antibodies, added to the DNA biotinylated probe, induced a supershift, suggesting that the bands detected by EMSA consisted not only of NF-κB subunits but of STAT3 protein as well. To further delineate this finding, we infected CLL cells with retroviral STAT3-shRNA and, using EMSA examined the binding of CLL cell nuclear extracts to the biotinylated NF-

κB p65 binding-site DNA probe. As shown in Fig. 4B, infection of CLL cells with retroviral STAT3-shRNA (previously found to downregulate STAT3 mRNA and protein levels (28)), but not with empty virus, significantly attenuated the binding of NF-κB heterodimers to DNA. To determine whether U-STAT3/NF-κB complexes bind to the promoters of NF-κB genes we used ChIP. As shown in Fig. 5A, anti-STAT3 antibodies coimmunoprecipitated DNA of STAT3, and the NF-κB-regulated genes VEGF C, CCL5 and CXCR5. Then, we asked whether U-STAT3/NF-κB complexes activate NF-κB-regulated genes. As shown in Fig. 5B, STAT3-shRNA downregulated mRNA levels of the NF-κB-regulated genes VEGF C, CCL5, and CXCR5 (which are not regulated by STAT3 (31, 32)) and, as previously reported (28), STAT3-shRNA downregulated STAT3 mRNA. Taken together, these data suggest that U-STAT3/NF-κB complexes bind to DNA and activate NF-κB-target genes.

Discussion

In the current study we show that NF-κB was constitutively activated in CLL cells from 15 patients with CLL, regardless of disease stage or treatment status. Several groups have demonstrated that NF-κB is activated in CLL (2-11) and that its activation is mediated by different, mostly extracellular, stimuli (4, 13-26). Usually, the activation of NF-κB is transient and cyclical in the presence of a continuous stimulus. For example, in mouse fibroblasts maintained by tumor necrosis factor (TNF), NF-κB-DNA binding appears and disappears every 30 to 60 minutes owing to repeated degradation and re-synthesis of IκB and the consequent activation and inactivation of NF-κB, respectively (33). Because NF-κB is constitutively activated in CLL, we assumed that the activation of NF-κB is most likely induced by an uninterrupted intracellular mechanism.

We recently found that CLL cells harbor high levels of U-STAT3, the production of which is induced by phosphoserine STAT3, constitutively upregulated in unstimulated CLL cells (28). STAT3 dynamically shuttles in and out of the nucleus independent of its phosphorylation status (34, 35). The shuttling of STAT3 from the cytoplasm to the nucleus requires binding of the coiled-coil domain of STAT3 to an importin-α-importin-β dimer to mediate passage through the nuclear pore complex. U-STAT3 also is constitutively exported out of the nucleus through a yet unknown mechanism. Similar to its phosphorylated forms, U-STAT3 can also affect NF-κB-regulated gene expression indirectly by binding to NF-κB and mediating its nuclear import (29, 36). Our data are in agreement with those observations. We found that U-STAT3 binds NF-κB and that the U-STAT3/NF-κB complex shuttles to the nucleus, binds to DNA, and activates NF-κB-regulated genes. Our data suggest that STAT3 plays a key role in the constitutive activation of NF-κB in CLL cells.

A broad array of stimuli activates NF-κB, including both endogenous and exogenous ligands and physical and chemical stresses (37). The protein components of pathways that culminate in IKK activation are not yet completely understood. Typically, the binding of cytokines such as TNF-α (38, 39) or interleukin-1 (40) to their corresponding cell surface receptors (e.g., TNF-receptor (TNFR) or a Toll-like receptor) recruits adaptor proteins (e.g., TNFR-associated factors (TRAFs) and receptor-interacting proteins) to the receptor's cytoplasmic domain, and these molecules, clustered at the receptor, activate the IKK complex. IKK then phosphorylates IκB at two serine residues, which leads to its ubiquitination and degradation

by the proteasome; NF-κB, free of its inhibitor, then enters the nucleus to turn on target genes (12). This established model appears to be more complex than initially thought. The crystal structure of IκBα bound to the p65/p50 heterodimer reveals that the IκBα protein masks only the nuclear localization sequence (NLS) of p65, whereas the NLS of p50 remains exposed. The exposed NLS of p50 coupled with nuclear export sequences (NES) in IκBα and p65 leads to constant shuttling of IκBα/NF-κB (p50/p65) complexes between the nucleus and the cytoplasm, despite steady state localization that appears almost exclusively cytosolic (30). Our confocal microscopy data showing NF-κB in the nucleus of normal B lymphocytes agree with this observation. Degradation of IκBα drastically alters the dynamic balance between cytosolic and nuclear localization signals to favor nuclear localization of NF-κB. Apparently, NF-κB might be activated without the degradation of IκB (29, 36), as we now show in CLL cells.

Several investigators have suggested that NF-κB should be considered a target for therapy in CLL (9-11). We have recently reported that STAT3-shRNA reduced STAT3 mRNA and protein levels and induced apoptosis in CLL cells (28). In the current study, we demonstrate that STAT3-shRNA inhibits the activity of NF-κB. Whether the STAT3 inhibitors currently being studied in clinical trials in CLL would also inhibit NF-κB activity remains to be determined.

Acknowledgments

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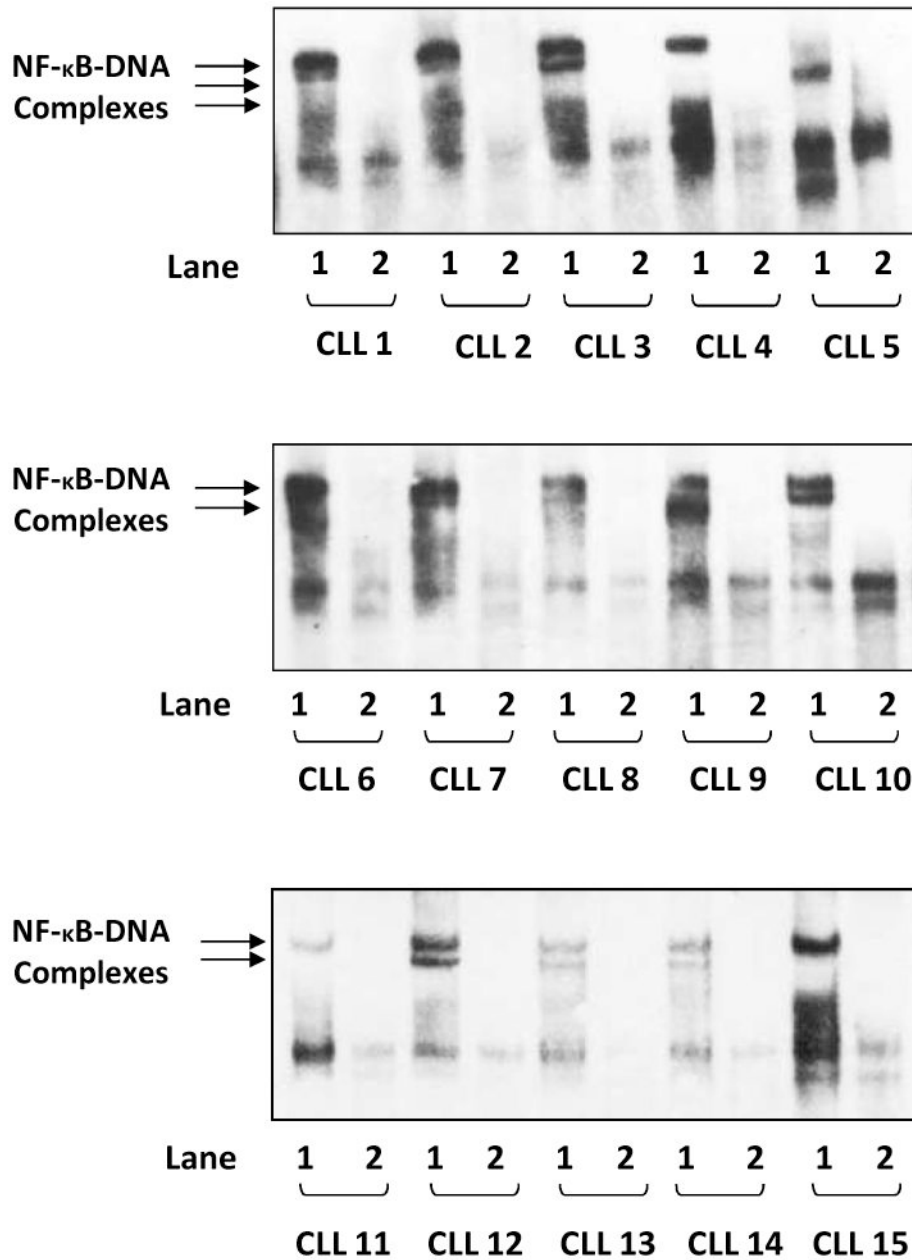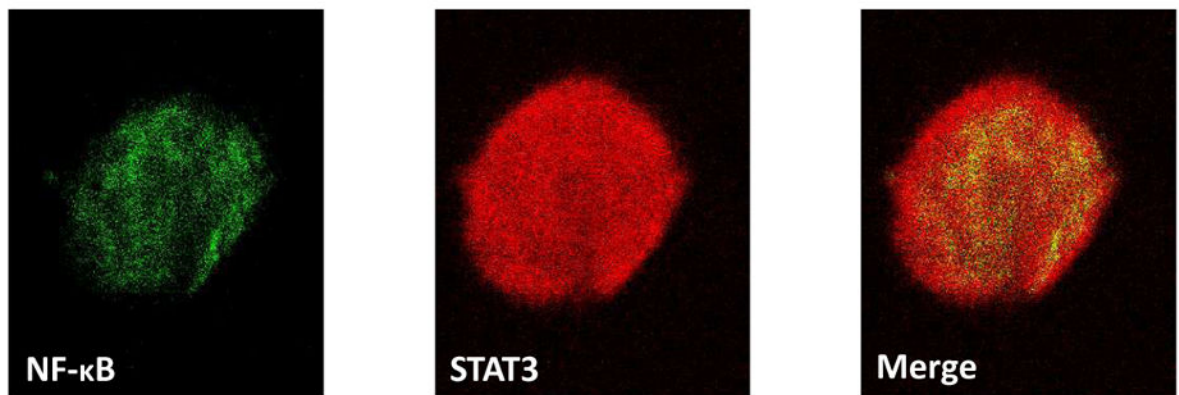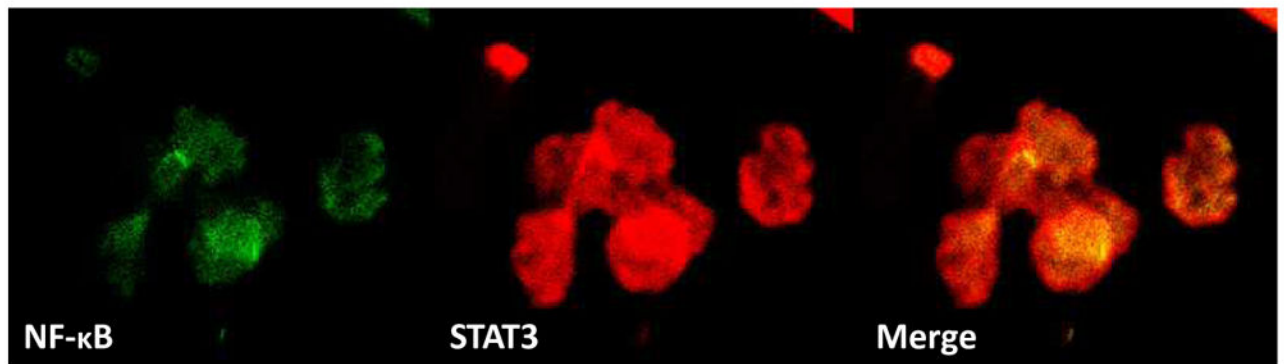
Figure 1.
NF-κB is constitutively activated in CLL cells. EMSA studies of PB low-density cell nuclear extracts from 15 different patients with CLL are presented. Lane 1 of each sample depicts binding of CLL cell nuclear extract to a biotin-labeled (hot) NF-κB DNA probe, whereas lane 2 depicts a reduction in binding when an excess of unlabeled probe was added to the biotin-labeled (hot) κB DNA probe (hot + cold). In all 15 samples, NF-κB-DNA (p50/p65) complexes were detected and NF-κB-DNA binding was almost completely abolished by the cold probe.
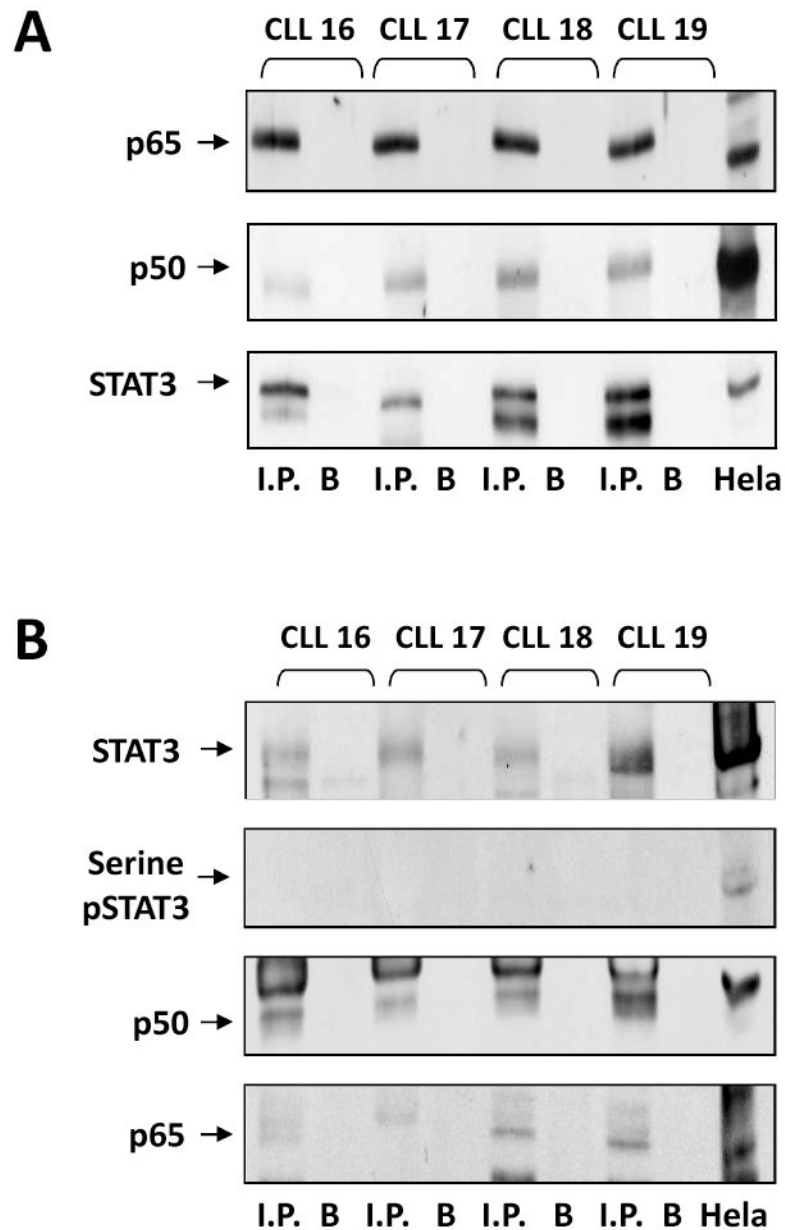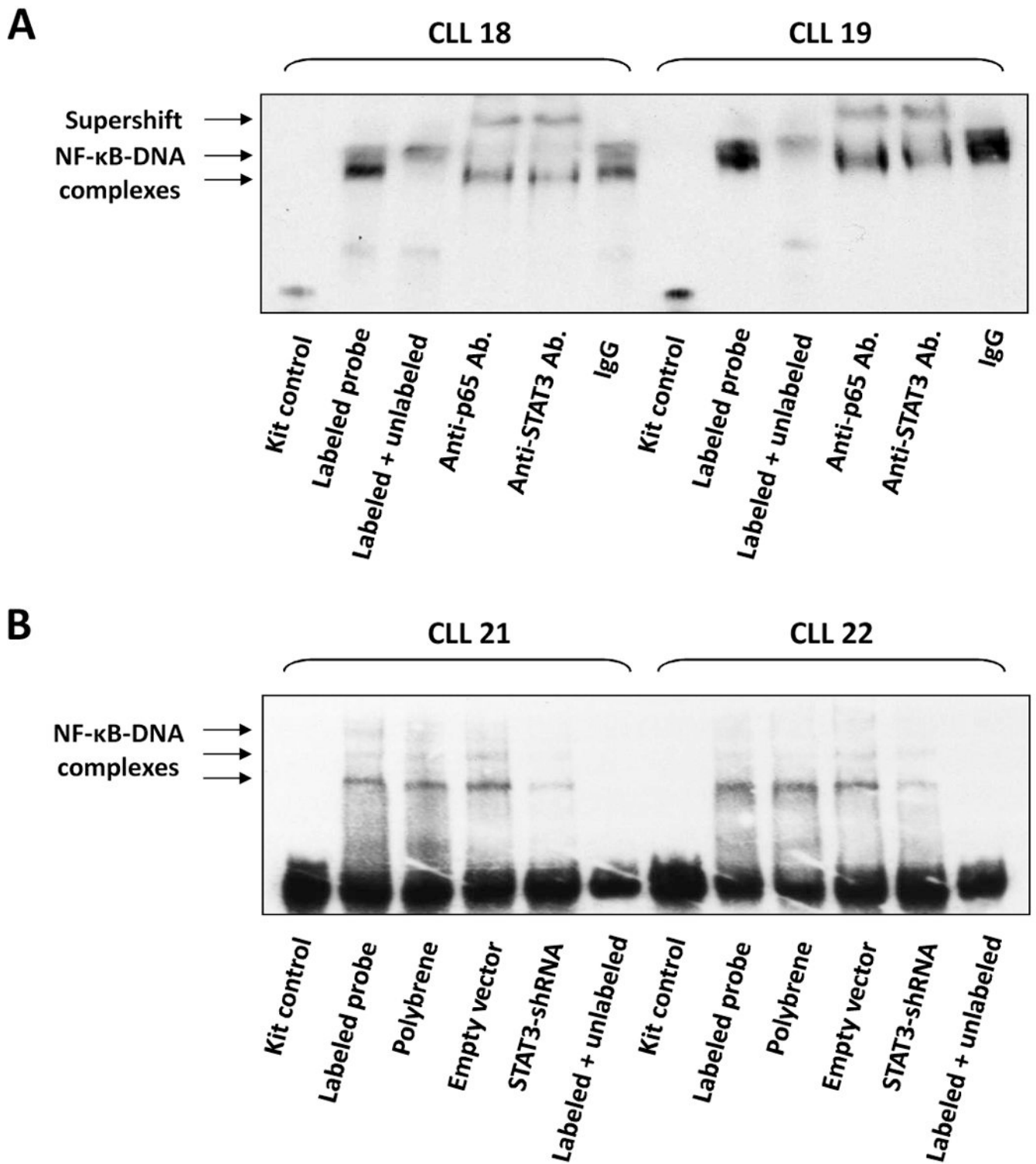
Figure 2.
U-STAT3 binds NF-κB p50/p65 dimers. **A.** PB low-density cell-protein extracts from four different patients with CLL were immunoprecipitated with anti-Stat3 antibodies. As shown, STAT3 and the NF-κB p65 and p50 proteins were detected in the immunoprecipitates of all three PB samples. **B.** PB low-density cell-protein extracts from the same patients with CLL were immunoprecipitated with anti-p65 antibodies. Hela cell protein extract was used as a positive control. As shown, p65, p50, STAT3, but not serine pSTAT3 proteins were detected in all immunopreciptates. I.P., immunoprecipitate; B, beads (control).

Figure 3.
U-STAT3-NF-κB complexes are detected in CLL cells but not in normal CD19+ cells. **A.**
Confocal microscopy studies of normal PB CD19+ cells detected a scattered signal of the
NF-κB p65 protein in the cytoplasm and nucleus (left panel; green), faint signals of STAT3
(middle panel; red) but no signal of STAT3-p65 complexes (right panel; yellow). **B.**
Confocal microscopy studies of PB CLL cells detected a dense signal of p65 (green),

STAT3 (red), and STAT3-p65 complexes (yellow) in the cytoplasm and mainly in the nucleus. One field of CLL cells (upper panel) and a single CLL cell (lower panel) are shown. Depicted are representative photomicrographs (X 400) obtained from two different samples of normal CD19+ lymphocytes and CLL cells of patients 19 and 20.
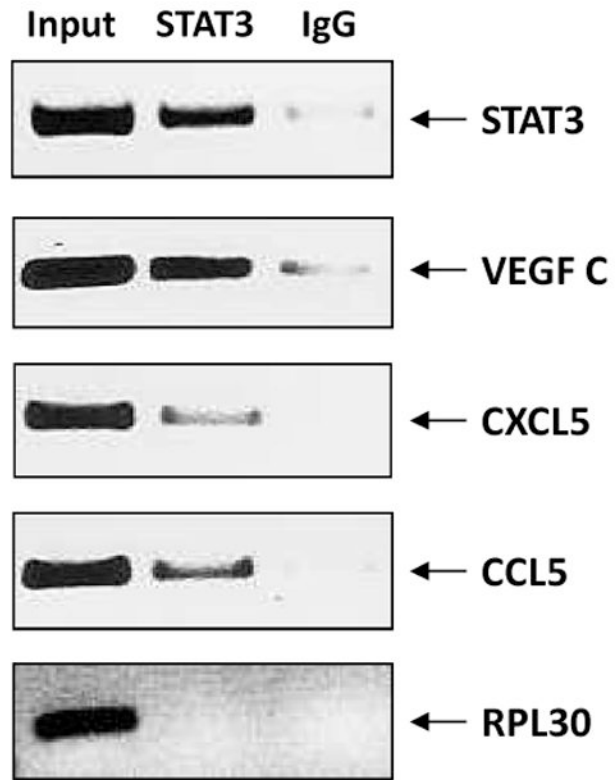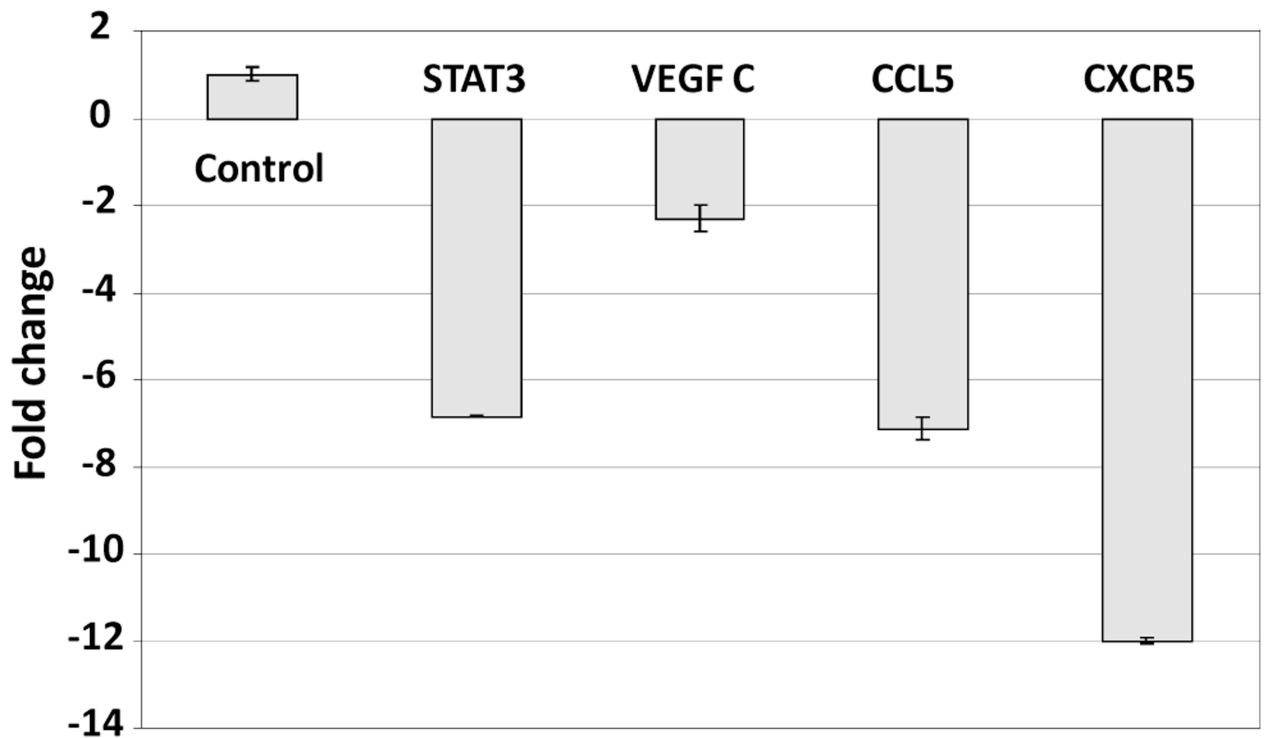
Figure 4.
The U-STAT3/NF-κB complex binds to DNA. **A.** CLL cell nuclear proteins p65 and STAT3
bind to a biotinylated κB-DNA probe. EMSA of CLL cell nuclear extract is depicted. As
shown, the addition of anti-p65 or anti-STAT3 antibodies to the κB DNA biotinylated probe

induced a supershift, suggesting that p65 and STAT3 bind to DNA. **B.** STAT3-shRNA attenuates the binding of CLL cell nuclear extract to a biotin-labeled DNA probe. CLL cells were infected either with lentiviral STAT3-shRNA or empty virus. Nuclear protein was extracted and EMSA was performed. As shown, NF-κB-DNA complexes were detected, and excess unlabeled (cold) DNA probe abolished the binding. Furthermore, infections with STAT3-shRNA, but not with empty virus, significantly attenuated NF-κB-DNA binding.

B

Figure 5.
U-STAT3/NF-κB complexes activate NF-κB-target genes. **A.** STAT3-shRNA downregulated mRNA levels of specific NF-κB-regulated genes. As shown, infection with lentiviral STAT3-shRNA downregulated mRNA levels of VEGF C, CCL5, and CXCR5. As expected, lentiviral STAT3-shRNA downregulated the level of STAT3 mRNA as well. In this experiment we used PB cells from patient 21. **B.** STAT3 co-immunoprecipitates with DNA of NF-κB-regulated genes. CLL cell-derived chromatin was immunoprecipitated by anti-STAT3 antibodies or rabbit serum (IgG control). The co-immunoprecipitated DNA was amplified by PCR using promoter constructs of STAT3, VEGF C, CCL5, CXCR5, or the control gene RPL30. As shown, DNA of STAT3, VEGF C, CCL5 and CXCR5 but not the control RPL30 gene, co-immunoprecipitated with STAT3 protein. STAT3, VEGF C, CCL5, or RPL30 DNA was detected in whole cell chromatin-extracted DNA (Input).

Table 1

Patient characteristics

CLL Pt. #	Sex	Age (Yr)	WBC (10⁹/l)	Lymph. %	Hb. (g/dl)	Plts. (10⁹/l)	Rai stage	CD38+/CD19+ %	β_2M (mg/dl)	V_H mutation	ZAP-70 %	Status	Cytogenetics	Previous treatment
1	M	58	73.7	90	13.1	128	1	NA	4.1	Y	80.9	Alive	t 12	
2	F	74	102.2	88	12.3	236	1	NA	1.9	N	NA	Alive	NA	
3	F	88	49.1	85	12.2	186	0	NA	3	ND	NA	Alive	D13	
4	M	61	52.9	84	14.8	129	1	0.9	2.7	N	Pos.	Alive	del 11q	FCR
5	M	63	143	82	13	164	1	97.5	3	Y	33.3	Alive	del 11q	FCR
6	F	70	118	94	12.5	178	1	62.5	3.2	Y	0	Alive	t 12	Revlimid
7	M	60	133.5	96	13.1	113	0	NA	2.8	N	0	Alive	del 13q	
8	F	56	95.5	97	13	334	0	2.1	2.4	Y	0	Alive	Dip.	
9	F	74	66.7	94	12.5	231	1	NA	1.8	ND	NA	Alive	NA	
10	F	75	160.7	90	12.1	146	1	1	4	Y	44.6	Alive	del 13q	FCR
11	F	72	136.6	91	11.8	244	2	2	3.6	Y	Pos.	Alive	t 12	Revlimid
12	M	63	96	92	14.7	155	0	0.3	3.5	Y	0	Alive	del 13q	
13	M	60	90.6	91	14.7	167	2	NA	4.8	Y	10.8	Alive	del 13q	FCR
14	F	70	26.4	93	10.1	101	3	0.2	3.2	Y	3.4	Alive	del 13q	Revlimid
15	F	58	172.6	98	13.3	212	1	NA	3.3	Y	0	Alive	NA	
16	F	72	46.9	79	9.8	272	3	2	4.7	Y	Pos.	Alive	t 12	Revlimid
17	F	60	111.8	92	12.7	219	0	NA	3.5	Y	1.8	Alive	NA	Ritux. GM
18	F	75	60.9	89	12.5	179	0	NA	3.5	Y	NA	Alive	NA	
19	M	47	130.9	88	13	258	1	98.8	3.3	Y	46.2	Alive	NA	FCR
20	M	48	8.7	20	15.2	173	0	NA	NA	N	Pos.	Alive	NA	CFAR
21	F	58	184.4	88	13.6	216	1	NA	2.7	N	Neg.	Alive	NA	
22	F	75	258.9	94	11.8	140	1	1.1	4.2	Y	44.6	Alive	del 13q	

Abbreviations: Yr., years; WBC, white blood cells; lymph., lymphocytes; Hb, hemoglobin; Plts., platelets; β_2M, β_2 microglobulin; M, male; F, female; N/A, not available; V_H mutation, hypermutation of the immunoglobulin heavy chain gene presented as N (negative; if % derivation from the germline sequence is ≤ 2%) or Y (positive, if % derivation from germline sequence is > 2%); Ritux., Rituxan; GM, granulocyte-macrophage colony-stimulating factor; FCR, cyclophosphamide, fludarabine, and rituxan; CFAR, cyclophosphamide, fludarabine, alemtuzumab, and rituxan; Pos., positive (analyzed by immunohistochemistry only); Neg., negative; t, translocation; del, deletion.